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Uncoupling between CD1d upregulation induced by retinoic acid and conduritol-B-epoxide and iNKT cell responsiveness

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ABSTRACT

Gaucher disease (GD) is associated with upregulation of CD1d and MHC-class II expression by monocytes. While the physiological impact of CD1d upregulation remains uncertain, it has been proposed that MHC-class II upregulation is associated with inflammation. Hereby, we show that the decrease in MHC-class II expression seen in GD patients under therapy correlates positively with chitotriosidase activity, a marker of inflamed macrophages. We also show that retinoic acid (RA) and the β-glucocerebrosidase inhibitor conduritol-B-epoxide (CBE) lead to upregulation of CD1d expression by THP-1 cells, which correlated with an increase in mRNA expression. *In vitro* co-culture experiments showed that RA treated THP-1 cells were more stimulatory for $CD4^+$ than for $CD8^+$ T cells, as determined by CFSE loss, in comparison to untreated THP-1 cells. Interestingly, even though addition of exogenous isoglobotrihexosylceramide (iGb3), a physiological CD1d ligand, augmented the percentage of dividing $CD4^+$ T cells, we could not detect a significant expansion of $CD4^+V\alpha24^+$ invariant Natural Killer T (iNKT) cells. In contrast, addition of α-galactosylceramide (α-GC) induced expansion of $V\alpha24^+$ iNKT cells as determined by using α-GC-loaded human CD1d dimers. These results strengthen the existence of a cross-talk between monocyte lipid accumulation, inflammation and changes in cell surface CD1d and MHC-class II in monocytes, which may result in inappropriate recognition events by immune cells and perpetuate chronic inflammation.

Introduction

Gaucher disease (GD), the most frequent lysosomal storage disorder (LSD) in Portugal (Pinto et al. 2004), results from the deficiency of the lysosomal hydrolase β-glucocerebrosidase (GluCerase), which is responsible for the hydrolysis of glucosylceramide (GluCer) into glucose and ceramide. Unmetabolized GluCer accumulates in lysosomes of macrophages that become engorged forming the so-called "Gaucher cells", the hallmark of the disease (Beutler and Grabowski 2001). GD is a multisystemic disorder with several clinical manifestations including hepatic, splenic and bone involvement. GD is characterized by peripheral inflammation that results from dysfunction of circulating or tissue-resident macrophages (Mehta 2006).

The catabolism of macromolecules in lysosomes is crucial for the correct functioning of the immune system as shown by several studies describing immune alterations in LSD (reviewed in Castaneda et al. 2008). Lipids are presented to particular T cell subsets, among them the invariant Natural Killer T (iNKT) cells, by CD1 molecules (Beckman et al. 1994; Kawano et al. 1997; Moody et al. 1997; Sieling et al. 1995). iNKT cells express an invariant T cell receptor formed by the canonical rearrangement of the Vα24 segment to the Jα18 segment associated with Vβ11 (Dellabona et al. 1993, 1994; Porcelli et al. 1993), and are characterized by the singular capacity of rapidly secreting cytokines upon activation when recognizing lipid antigens in the context of the CD1d antigen-presenting molecule (Godfrey et al. 2000). CD1d molecules are structurally similar to MHC-class I molecules, but intracellularly behave like MHC-class II molecules (Porcelli

Abbreviations: GD, Gaucher disease; RA, retinoic acid; CBE, conduritol-B-epoxide; GluCerase, β-glucosylcerebrosidase; GluCer, glucosylceramide; LSD, lysosomal storage disorders; iNKT cells, invariant natural killer T cells; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood leukocytes; ERT, enzyme replacement therapy; PBS, phosphate-buffered saline solution; BSA, bovine serum albumin; NaN_3, sodium azide; MFI, mean fluorescence intensity; 4-MU, 4-methylumbelliferone; iFBS, heat-inactivated foetal bovine serum; RT-PCR, reverse transcription polymerase chain reaction; iGb3, isoglobotrihexosylceramide; CFSE, carboxyfluorescein succinimidyl ester; α-GC, α-galactosylceramide; ST, lipid standards; PE, phosphatidylethanolamine; LacCer, lactosylceramide; Gb3, globotrihexosylceramide; Sph, sphingomyelin; Cer 4, ganglioside GM1

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and Modlin 1999) in that most CD1d molecules are directed from the endoplasmic reticulum to the plasma membrane and from where they are internalized to the endocytic compartments. Some CD1d molecules are found associated with the invariant chain and are directed to MHC-class II compartments (Kang and Cresswell 2002).

We have shown in a previous study that monocytes from GD patients presented upregulation of CD1d and MHC-class II, and we hypothesized that CD1d upregulation resulted from alterations in intracellular trafficking as a consequence of lipid accumulation, while MHC-class II upregulation appeared to be related to the inflammatory status of the patients (Balreira et al. 2005). We also reported on upregulation of MHC-class II, but not CD1d, in monocytes from another LSD, Fabry disease (Balreira et al. 2008). The association of CD1d upregulation with sphingolipid accumulation was further supported by results obtained with peripheral blood mononuclear cells (PBMC) from healthy controls treated with an irreversible inhibitor of GluCerase, conduritol-B-epoxide (CBE) (Balreira et al. 2005). Nevertheless, whether alterations in CD1d and MHC-class II due to sphingolipid accumulation underlie imbalances in regulatory iNKT and T cells and contribute to the heterogeneity of the clinical manifestations remains to be elucidated. In order to address these questions, the development of *in vitro* models of the Gaucher cell (a lipid-laden macrophage) could be an important tool to dissect the molecular mechanism responsible for the upregulation of CD1d/MHC-II and ascertain whether this upregulation has any impact on conventional and invariant T cell populations. In this study, we report on the impact that an *in vitro* Gaucher cell model, a "lipid-laden" macrophage-like cell line, has on T cell responsiveness.

Material and methods

Patient population

GD patients were diagnosed through the determination of residual activity of GluCerase in peripheral blood leukocytes (PBL) (Sa Miranda et al. 1990). Patients were genotyped using previously described methods and most of their genotypes have already been reported (Amaral et al. 1999, 1993, 2000, 1996). The study included healthy controls, untreated GD patients (GD patients) and GD patients submitted to enzyme replacement therapy (ERT) with imiglucerase. Treatment was initiated with 60 U/kg every 2 weeks and adjusted according to the patient's clinical response. All studies were performed with the patients' consent and with the approval of the Ethical Committee of Instituto de Biologia Molecular e Celular.

Expression of cell surface molecules

Cell surface staining of peripheral mononuclear and THP-1 cells was performed on ice for 30 min in the dark in phosphate-buffered saline (PBS) solution supplemented with 0.1% sodium azide (NaN_3) and 0.2% bovine serum albumin (BSA) (PBS/BSA/NaN_3, staining solution) in round-bottom microtiter plates (Greiner-Nurtingen, Germany) with $\sim 0.5 \times 10^6$ cells/well. After the staining, cells were washed twice with staining solution and immediately analyzed in a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Monocytes were identified and gated according to FSC/SSC characteristics. For peripheral blood samples, 50,000 events within the mononuclear gate were acquired, and for THP-1 cells, 20,000 events were acquired and analyzed using the FlowJo software (Tree Star, Inc, Ashland, USA). The following fluorochrome-conjugated monoclonal antibodies were used: anti-HLA-A, B, C (clone W6/32) from Immunotools (Friesoythe, Germany); anti-HLA-DR, DP, DQ (clone CR3/43) from DakoCytomation (Glostrup, Denmark); anti-CD1d (clone 42) from BD Biosciences (Erembodegem, Belgium) and anti-CD1d (clone Nor 3.2), from AbD Serotec (Oxford, UK). Mouse immunoglobulins were used as isotype controls. Expression of the different molecules at the cell surface was determined by mean fluorescence intensity (MFI) values.

Determination of chitotriosidase activity

Chitotriosidase activity was measured as previously described (Hollak et al. 1994). Briefly, 5 μL of plasma were incubated with 100 μL of 0.026 mM 4-MU-β-D-N-N′-N″-triacetilchitotriosidase (Sigma-Aldrich, St. Louis, MO, USA) as substrate in 50 mM citrate, 100 mM phosphate, pH 5.2, at 37 °C, for 15 min. After incubation, the reaction was stopped with 1 mL of 1 M glycine/NaOH buffer, pH 10.0. Fluorescent 4-methylumbelliferone (4-MU) was measured at 445 nm with a fluorescence spectrophotometer (Hitachi HTA, Illinois, USA). Whenever necessary, samples were previously diluted 50 × in BSA 1%.

Cells

PBMC were obtained from leukocyte preparations of healthy subjects by centrifugation over the density gradient Lymphoprep (Axis-Shield, Oslo, Norway). Contaminating red blood cells were lysed in lysis solution (10 mM Tris, 150 mM NH_4Cl, pH 7.4) for 10 min at 37 °C. PBMC were kept in culture overnight in Petri dishes (Orange Scientific, Braine-l'Alleud, Belgium) in RPMI-1640 with Glutamax I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 1% penicillin/streptomycin/amphotericin B (P/S/A), 5% heat-inactivated foetal bovine serum (iFBS) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C, 5% CO_2, 99% humidity. After overnight culture, non-adherent cell suspensions (PBL) were recovered for co-culture assays. Pure $CD4^+$ and $CD8^+$ T cells were obtained from PBL, by positive selection using MACS microbeads and columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolation of human iNKT cell clones from PBMC of healthy donors was performed as described before (Franchini et al. 2007). The monocytic leukemia cell line THP-1 (ECACC, Salisbury, UK) was kept in culture and grown in RPMI-1640 with Glutamax I complete medium with 1% P/S/A and 10% iFBS, at 37 °C, 5% CO_2, 99% humidity.

Inhibition of GluCerase activity in THP-1 cells

GluCerase activity was inhibited as described (Balreira et al. 2005). Briefly, THP-1 cells were cultured for 96 h in normal media or media supplemented with 1 mM of CBE (Biomol, Plymouth Meeting, PA, USA), prepared as stock solution in 20 mM Hepes buffer, pH 7.4 (Korkotian et al. 1999). Cells were then harvested, washed and processed for flow cytometry or thin layer chromatography. Cells processed for flow cytometry were acquired in a FACScalibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using the FlowJo software (Tree Star Inc., Ashland, USA).

Thin layer chromatography (TLC)

After sonication of cell samples, total lipids were extracted by two cycles of 1 min vortexing with 4.5 mL chloroform/methanol (1:2, v/v). The extract was evaporated under a stream of nitrogen at 40 °C. Total lipids were fractionated into acidic and neutral lipids by polarity differences. Thus, dried total lipids were dissolved in chloroform/methanol/water (2:8:4, v/v/v)